SureSelect^{XT} Target Enrichment System for Illumina Paired-End Sequencing Library

Illumina HiSeq and MiSeq Multiplexed Sequencing Platforms

Protocol

Version B.2, April 2015

SureSelect platform manufactured with Agilent SurePrint Technology

For Research Use Only. Not for use in Diagnostic Procedures.

Before you begin, view hands-on videos of SureSelect procedures at
http://www.agilent.com/genomics/protocolvideos.

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Manual Part Number

G7530-90000

Edition

Version B.2, April 2015

Printed in USA

Agilent Technologies, Inc.
5301 Stevens Creek Blvd
Santa Clara, CA 95051 USA

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Notice to Purchaser

SureSelect capture libraries and reagents must be used within one year of receipt.

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CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

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In this Guide...

This guide provides an optimized protocol for Illumina paired-end multiplexed library preparation using the SureSelect^{XT} Library Prep and Capture System.

This guide describes the recommended operational procedures to capture the genomic regions of interest using biotinylated RNA oligomer libraries to enrich targeted regions of the genome from repetitive sequences and sequences unrelated to the research focus prior to sample sequencing using the Illumina platform.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation (3 µg DNA Samples)

This chapter describes the steps to prepare libraries for target enrichment from 3-µg gDNA samples.

3 Sample Preparation (200 ng DNA Samples)

This chapter describes the steps to prepare libraries for target enrichment from 200-ng gDNA samples.

4 Hybridization and Capture

This chapter describes the steps to hybridize and capture the prepared library DNA.

5 Indexing and Sample Processing for Multiplexed Sequencing

This chapter describes the steps to amplify, purify, and assess quality and quantity of the sample libraries. Samples are pooled by mass prior to sequencing.

6 Reference

This chapter contains reference information, including component kit contents and index sequences.

What's New in Version B.2

- Support for the OneSeq Capture Libraries (see Table 4 on page 16 and Table 40 on page 73).
- Support for ClearSeq Capture Libraries, including ClearSeq Comprehensive Cancer XT Libraries (see Table 4 on page 16).

What's New in Version B.1

- Support for kits with either 8-bp indexes A01 to H12 (revised index configuration, typically received December 2014 or later) or 6-bp indexes 1 to 16 (original index configuration, typically received before December 2014).

  Kits with 8-bp indexes include indexing primers provided in white-capped tubes (16 Reactions) or blue plate (96 Reactions). For indexing protocol details, see page 70. For kit content details see page 88. For nucleotide sequences of the 8-bp indexes, see page 92.

  Kits with 6-bp indexes include indexing primers provided in clear-capped tubes. For indexing protocol details, see page 74. For kit contents see page 93. For nucleotide sequences of the 6-bp indexes, see page 96.
- Support for sample processing in 96-well plates or 8-well strip tubes. See Table 6 on page 17 for recommended thermal cycler and plasticware. Protocol modifications for plate/strip tube sample processing are located throughout this guide. Key modifications include reduction in wash solution volumes to 200 µL including ethanol washes (for example, see step 8 on page 24) and capture washes (for example, see step 6 on page 67).
- Revision of hybridization setup instructions (see page 60 through page 65). Revisions do not affect the final composition of the hybridization reaction, but format and order of certain preparation steps have been modified.
- Removal of SureSelect Elution Buffer and Neutralization Buffer from SureSelect Target Enrichment Box 1 (p/n 5190-8645 or 5190-8646; see Table 49 on page 90).

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Make sure you have the most current protocol. Go to genomics.agilent.com and search for G7530-90000.

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

NOTE This protocol differs from the Illumina Multiplexed Paired-End sequencing manual and other SureSelect protocols at several steps. Pay close attention to the primers used for each amplification step and the blocking agents used during hybridization.

NOTE Agilent cannot guarantee the SureSelect Target Enrichment kits and cannot provide technical support for the use of non-Agilent protocols or instruments to process samples for enrichment.

Overview of the Workflow

The SureSelect^{XT} target enrichment workflow is summarized in Figure 1. The estimated time requirements for each step are summarized in Table 1.

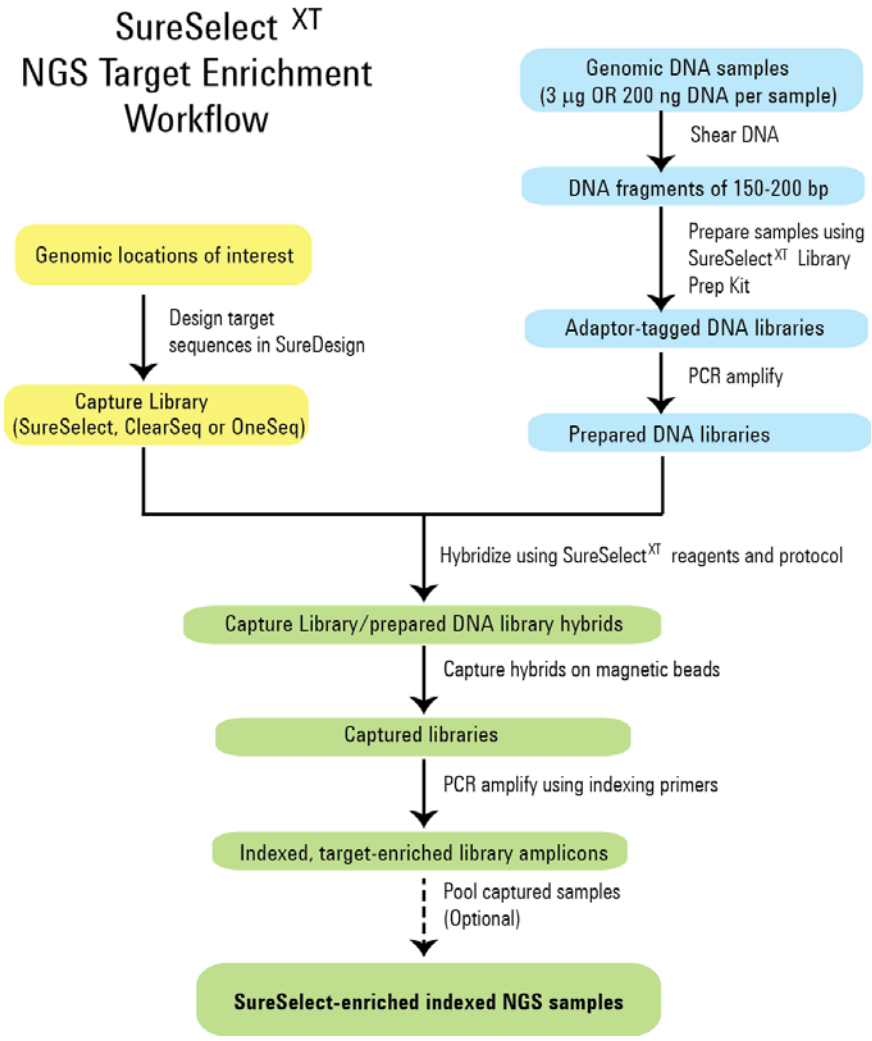

Figure 1 Overall target-enriched sequencing sample preparation workflow.

Table 1 Estimated time requirements

Step	Time
Prepped Library Production	1 day
Library Hybridization	16 or 24 hours
Streptavidin bead preparation	30 minutes
Hybrid capture and washing	2 hours
DNA purification	30 minutes
Post-capture indexing and amplification	1 hour
PCR product purification	30 minutes
QC using Bioanalyzer or TapeStation	2 to 3 hours
Pooling of indexed samples by mass	< 1 hour

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Use best-practices to prevent PCR product contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
  2. Maintain clean work areas. Clean pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
  3. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
  4. Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
- Do not mix stock solutions of gDNA on a vortex mixer. Instead, gently tap the tube with your finger to mix the sample.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as rapidly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store on ice or in a cold block until use.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of domed caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- In general, follow Biosafety Level 1 (BL1) safety rules.
- Possible stopping points, where samples may be stored at –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

Safety Notes

CAUTION

- Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Required Reagents

Table 2 Required Reagents for SureSelect^{XT} Target Enrichment

Description	Vendor and part number
SureSelect, ClearSeq or OneSeq Capture Library*	Select the appropriate library from Table 3 or Table 4
SureSelect^{XT} Reagent Kit, Illumina (ILM) platforms*	Agilent
HiSeq platform, 16 reactions	p/n G9611A
HiSeq platform, 96 reactions	p/n G9611B
HiSeq platform, 480 reactions	p/n G9611C
MiSeq platform, 16 reaction	p/n G9612A
MiSeq platform, 96 reactions	p/n G9612B
MiSeq platform, 480 reactions	p/n G9612C
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
450 mL	p/n A63882
Herculase II Fusion DNA Polymerase (includes dNTPs and 5× Buffer)	Agilent
200 Reactions (processes 100 XT libraries)	p/n 600677
400 Reactions	p/n 600679
Dynabeads MyOne Streptavidin T1	Life Technologies
2 mL	p/n 65601
10 mL	p/n 65602
100 mL	p/n 65603
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Life Technologies p/n 12090-015, or equivalent
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Qubit dsDNA HS Assay Kit *or*	Life Technologies p/n Q32851
Qubit BR dsDNA Assay Kit	Life Technologies
100 assays	p/n Q32850
500 assays	p/n Q32853
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930

* SureSelect, ClearSeq, and OneSeq reagents must be used within one year of receipt.

Table 3 SureSelect^{XT} Capture Libraries*

Capture Library	16 Reactions	96 Reactions	480 Reactions
SureSelect^{XT} Clinical Research Exome	5190-7338	5190-7339	–
SureSelect^{XT} Focused Exome	5190-7787	5190-7788	–
SureSelect^{XT} Focused Exome Plus 1	5190-7790	5190-7791	–
SureSelect^{XT} Focused Exome Plus 2	5190-7793	5190-7795	–
SureSelect^{XT} Human All Exon v5	5190-6208	5190-6209	–
SureSelect^{XT} Human All Exon v5 + UTRs	5190-6213	5190-6214	–
SureSelect^{XT} Human All Exon v5 + lncRNA	5190-6446	5190-6447	–
SureSelect^{XT} Human All Exon v5 Plus	5190-6211	5190-6212	–
SureSelect^{XT} Human All Exon v4	5190-4631	5190-4632	5190-4634
SureSelect^{XT} Human All Exon v4 + UTRs	5190-4636	5190-4637	5190-4639
SureSelect^{XT} Mouse All Exon	5190-4641	5190-4642	5190-4644
SureSelect^{XT} Human X-Chromosome	5190-4651	5190-4652	5190-4653
SureSelect^{XT} Custom 1 kb up to 499 kb (reorder)	5190-4806 (5190-4811)	5190-4807 (5190-4812)	5190-4809 (5190-4814)
SureSelect^{XT} Custom 0.5 Mb up to 2.9 Mb (reorder)	5190-4816 (5190-4821)	5190-4817 (5190-4822)	5190-4819 (5190-4824)
SureSelect^{XT} Custom 3 Mb up to 5.9 Mb (reorder)	5190-4826 (5190-4831)	5190-4827 (5190-4832)	5190-4829 (5190-4834)
SureSelect^{XT} Custom 6 Mb up to 11.9 Mb (reorder)	5190-4836 (5190-4841)	5190-4837 (5190-4842)	5190-4839 (5190-4844)
SureSelect^{XT} Custom 12 Mb up to 24 Mb (reorder)	5190-4896 (5190-4901)	5190-4897 (5190-4902)	5190-4899 (5190-4904)

* SureSelect capture libraries must be used within one year of receipt.

Table 4 Compatible ClearSeq and OneSeq Capture Libraries

Capture Library	16 Reactions	96 Reactions	480 Reactions
ClearSeq Comprehensive Cancer XT	5190-8011	5190-8012	–
ClearSeq Comprehensive Cancer Plus XT	5190-8014	5190-8015	–
ClearSeq Inherited Disease XT	5190-7518	5190-7519	–
ClearSeq Inherited Disease Plus XT	5190-7521	5190-7522	–
ClearSeq DNA Kinome XT	5190-4646	5190-4647	5190-4649
OneSeq Constitutional Research Panel	5190-8702	5190-8703	–
OneSeq Hi Res CNV Backbone + Custom 1–499 kb	5190-8705	5190-8887	–
OneSeq Hi Res CNV Backbone + Custom 0.5 –2.9 Mb	5190-8889	5190-8890	–
OneSeq Hi Res CNV Backbone + Custom 3–5.9 Mb	5190-8892	5190-8893	–
OneSeq Hi Res CNV Backbone + Custom 6–11.9 Mb	5190-8895	5190-8896	–

Optional Reagents

Table 5 Optional Reagents for SureSelect^{XT} Target Enrichment

Description	Vendor and part number
Agilent QPCR NGS Library Quantification Kit (Illumina GA)	Agilent p/n G4880A
Ethylene Glycol	American Bioanalytical p/n AB00455
Tween 20	Sigma-Aldrich p/n P9416-50ML

Required Equipment

Table 6 Required Equipment for SureSelect^{XT} Target Enrichment

Description	Vendor and part number
SureCycler 8800 Thermal Cycler, or equivalent	Agilent p/n G8800A
96 well plate module for SureCycler 8800 Thermal Cycler	Agilent p/n G8810A
SureCycler 8800-compatible plasticware:	
96-well plates	Agilent p/n 410088
OR	
8-well strip tubes	Agilent p/n 410092
Tube cap strips, domed	Agilent p/n 410096
DNA Analysis Platform and Consumables	
Agilent 2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA
Agilent 2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA
Agilent DNA 1000 Kit	Agilent p/n 5067-1504
Agilent High Sensitivity DNA Kit	Agilent p/n 5067-4626
OR	
Agilent 2200 TapeStation	Agilent p/n G2964AA or G2965AA
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
Qubit Fluorometer	Life Technologies p/n Q32857
Qubit Assay Tubes	Life Technologies p/n Q32856
Covaris Sample Preparation System, S-series or E-series model	Covaris
Covaris sample holders	
96 microTUBE plate (E-series only)	Covaris p/n 520078
microTUBE for individual sample processing	Covaris p/n 520045
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent

Table 6 Required Equipment for SureSelect^{XT} Target Enrichment

Description	Vendor and part number
Microcentrifuge	Eppendorf microcentrifuge model 5417C or equivalent
Plate or strip tube centrifuge	Labnet International MPS1000 Mini Plate Spinner p/n C1000 (requires adapter, p/n C1000-ADAPT, for use with strip tubes) or equivalent
Nutator plate mixer	BD Diagnostics p/n 421105, or equivalent
Magnetic separator	Life Technologies p/n 12331D or equivalent*
Multichannel pipette	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vortex mixer	
Vacuum concentrator	Savant SpeedVac, model DNA120, or equivalent
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	

* Select a magnetic separator configured to collect magnetic particles on one side of each well. Do not use a magnetic separator configured to collect the particles in a ring formation.

Optional Equipment

Table 7 Optional Equipment for SureSelect^{XT} Target Enrichment

Description	Vendor and part number
Tube-strip capping tool	Agilent p/n 410099
PlateLoc Thermal Microplate Sealer with Small Hotplate	Agilent p/n G5402A
Peelable Aluminum Seal for PlateLoc Sealer	Agilent p/n 24210-001
Mx3005P Real-Time PCR System	Agilent p/n 401449 or equivalent
Mx3000P/Mx3005P 96-well tube plates	Agilent p/n 410088 or equivalent
Mx3000P/Mx3005P optical strip caps	Agilent p/n 401425 or equivalent
MicroAmp Clear Adhesive Film	Life Technologies p/n L12-20

2 Sample Preparation (3 µg DNA Samples)

Step 1. Shear the DNA 22
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Step 9. Purify the sample using AMPure XP beads 33
Step 10. Amplify the adaptor-ligated library 34
Step 11. Purify the amplified library with AMPure XP beads 37
Step 12. Assess quality and quantity 38

CAUTION This section contains instructions for the preparation of gDNA libraries from 3 µg DNA samples. **For lower input (200 ng) DNA samples, see the library preparation protocol on page 41.**

The sample preparation protocol is used to prepare DNA libraries for sequencing using the Illumina paired-read platform. For each sample to be sequenced, an individual indexed library is prepared. For an overview of the SureSelect^{XT} target enrichment workflow, see Figure 1 on page 10.

The steps in this section differ from the Illumina protocol in the use of the Covaris system for gDNA shearing, smaller target shear size, elimination of size selection by gel purification, implementation of AMPure XP beads for all purification steps, and primers used for PCR. Refer to the Illumina protocol *Preparing Samples for Multiplexed Paired-End Sequencing* (p/n1005361) or the appropriate Illumina protocol for more information.

Step 1. Shear the DNA

NOTE Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0. Use the Qubit system to quantify genomic DNA before library preparation.

For each DNA sample to be sequenced, prepare 1 library.

1 Set up the Covaris E-series or S-series instrument.

   a Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate in use.

   b Check that the water covers the visible glass part of the tube.

   c On the instrument control panel, push the Degas button. Degas the instrument for least 30 minutes before use, or according to the manufacturer's recommendations.

   d Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C.

   e *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

   Refer to the Covaris instrument user guide for more details.

2 Put a Covaris microTube into the loading and unloading station.

   Keep the cap on the tube.

NOTE You can use the 96 microTube plate (see Table 6 on page 17) for the DNA shearing step when preparing multiple gDNA samples in the same experiment.

3 Use the Qubit dsDNA Assay to determine the concentration of your gDNA sample.

   Follow the instructions for the instrument.

4 Dilute 3 µg of high-quality gDNA with 1X Low TE Buffer in a 1.5-mL LoBind tube to a total volume of 130 µL.

5 Use a tapered pipette tip to slowly transfer the 130-µL DNA sample through the pre-split septa.

   Be careful not to introduce a bubble into the bottom of the tube.

6 Secure the microTube in the tube holder and shear the DNA with the settings in Table 8 or Table 9, depending on the Covaris instrument SonoLab software version used.

The target DNA fragment size is 150 to 200 bp.

Table 8 Shear settings for Covaris instruments using SonoLab software version 7 or newer

Setting	Value
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	200
Treatment Time	360 seconds
Bath Temperature	4° to 8° C

Table 9 Shear settings for Covaris instruments using SonoLab software prior to version 7

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Time	6 cycles of 60 seconds each
Set Mode	Frequency sweeping
Temperature	4° to 7° C

7 Put the Covaris microTube back into the loading and unloading station.

8 While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA.

9 Transfer each sheared DNA sample (approximately 130 µL) to a separate well of a 96-well plate or strip tube.

Step 2. Purify the sample using AMPure XP beads

NOTE Instructions in this protocol are for sample processing in 96-well PCR plates. When processing a small number of samples, you can instead use strip tubes or individual tubes that are compatible with the thermal cycler and magnetic separation device used in the protocol.

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.

NOTE The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 2 mL of fresh 70% ethanol per sample.

3. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 180 µL of homogeneous AMPure XP beads to each sheared DNA sample (approximately 130 µL) in the PCR plate. Pipette up and down 10 times to mix.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
7. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.

NOTE If some magnetic beads remain suspended in solution after 5 minutes, carefully remove and discard 100 µl of cleared solution from near the bottom of the wells, and continue incubating the plate in the magnetic stand for an additional 3 minutes. After the remaining suspension has cleared, remove and discard the remaining cleared solution (approximately 210 µl) from the wells.

8. Continue to keep the plate in the magnetic stand while you dispense 200 µL of 70% ethanol in each sample well.

Use fresh 70% ethanol for optimal results.

9 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.

10 Repeat step 8 to step 9 once.

11 Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

12 Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 3 to 5 minutes or until the residual ethanol completely evaporates.

NOTE Do not dry the bead pellet to the point that the pellet appears cracked during any of the bead drying steps in the protocol. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

13 Add 50 µL nuclease-free water to each sample well.

14 Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.

15 Incubate for 2 minutes at room temperature.

16 Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

17 Remove the cleared supernatant (approximately 48 µL) to a fresh PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 3. Assess quality (optional)

This step is optional.

Quality assessment can be done with either the 2100 Bioanalyzer instrument or the 2200 TapeStation instrument.

Option 1: Analysis using the 2100 Bioanalyzer and DNA 1000 Assay

Use a DNA 1000 chip and reagent kit for analysis of the 3 µg sheared DNA samples using the 2100 Bioanalyzer. See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the DNA 1000 assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Check that the electropherogram shows a DNA fragment size peak between 150–200 bp. A sample electropherogram is shown in Figure 2.

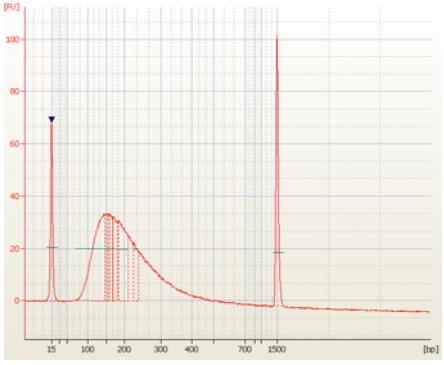

Figure 2 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using the 2200 TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and D1000 Reagents for analysis of the 3 µg sheared DNA samples using the 2200 TapeStation. For more information to do this step, see the *Agilent 2200 TapeStation User Manual* at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the *Agilent 2200 TapeStation User Manual*. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the D1000 ScreenTape, and loading tips into the 2200 TapeStation as instructed in the *Agilent 2200 TapeStation User Manual*. Start the run.

3 Verify that the electropherogram shows a DNA fragment size peak between 150–200 bp. A sample electropherogram is shown in Figure 3.

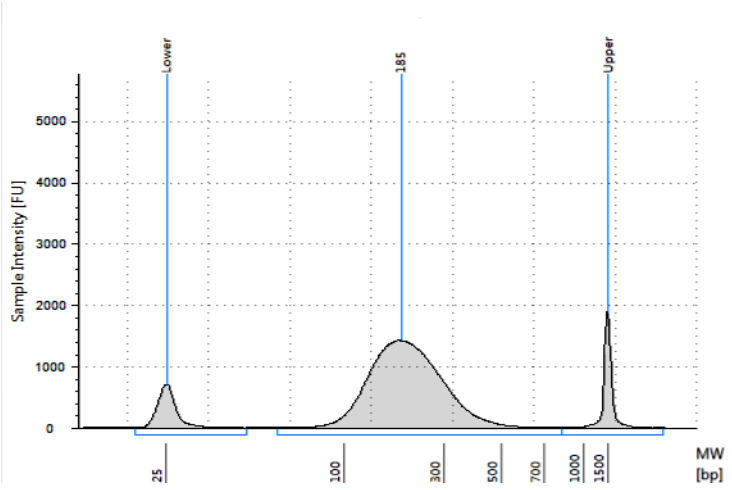

Figure 3 Analysis of sheared DNA using a D1000 ScreenTape.

Step 4. Repair the ends

Use the SureSelect XT Library Prep Kit ILM for this step.

To process multiple samples, prepare master mixes with overage at each step, without the DNA sample. Master mixes for preparation of 16 samples (including excess) are shown in each table as an example.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of End Repair master mix, as described in Table 10, on ice. Mix well on a vortex mixer.

Table 10 Preparation of End Repair master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	35.2 µL	580.8 µL
10× End Repair Buffer (clear cap)	10 µL	165 µL
dNTP Mix (green cap)	1.6 µL	26.4 µL
T4 DNA Polymerase (purple cap)	1 µL	16.5 µL
Klenow DNA Polymerase (yellow cap)	2 µL	33 µL
T4 Polynucleotide Kinase (orange cap)	2.2 µL	36.3 µL
Total	**52 µL**	**858 µL**

2 Add 52 µL of the master mix to each PCR plate sample well containing purified, sheared DNA. Mix by pipetting up and down.

3 Incubate the plate in the thermal cycler and run the program in Table 11. Do not use a heated lid.

Table 11 End-Repair Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

Step 5. Purify the sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 180 µL of homogeneous AMPure XP beads to each 100-µL end-repaired DNA sample in the PCR plate. Pipette up and down 10 times to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 to step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 3 to 5 minutes or until the residual ethanol completely evaporates.
12. Add 32 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 30 µL) to a fresh PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 6. Adenylate the 3' end of the DNA fragments

Use the SureSelect XT Library Prep Kit ILM for this step.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of Adenylation master mix, as described in Table 12, on ice. Mix well on a vortex mixer.

Table 12 Preparation of Adenylation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	11 µL	181.5 µL
10× Klenow Polymerase Buffer (blue cap)	5 µL	82.5 µL
dATP (green cap)	1 µL	16.5 µL
Exo(–) Klenow (red cap)	3 µL	49.5 µL
Total	**20 µL**	**330 µL**

2 Add 20 µL of the Adenylation master mix to each end-repaired, purified DNA sample (approximately 30 µL).

3 Mix well by pipetting up and down.

4 Incubate the plate in the thermal cycler and run the program in Table 13. Do not use a heated lid.

Table 13 dA-Tailing Thermal Cycler Program

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	4°C	Hold

Step 7. Purify the sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL dA-tailed DNA sample in the PCR plate. Pipette up and down 10 times to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 to step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 15 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove 13 µL of the cleared supernatant to a fresh PCR plate well. You can discard the beads at this time.
17. Proceed immediately to the next step, Step 8. Ligate the paired-end adaptor.

Step 8. Ligate the paired-end adaptor

Use the SureSelect XT Library Prep Kit ILM for this step.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of Ligation master mix, as described in Table 14, on ice. Mix well on a vortex mixer.

Table 14 Preparation of Ligation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	15.5 µL	255.75 µL
5× T4 DNA Ligase Buffer (green cap)	10 µL	165 µL
SureSelect Adaptor Oligo Mix (brown cap)	10 µL	165 µL
T4 DNA Ligase (red cap)	1.5 µL	24.75 µL
Total	**37 µL**	**610.5 µL**

2 Add 37 µL of the Ligation master mix to each dA-tailed, purified DNA sample (13 µL) in the PCR plate wells.

3 Mix well by pipetting up and down.

4 Incubate the plate in the thermal cycler and run the program in Table 15. Do not use a heated lid.

Table 15 Ligation Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	4°C	Hold

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 9. Purify the sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each adaptor-ligated DNA sample in the PCR plate (50 µL). Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 32 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 32 µL) to a fresh PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 10. Amplify the adaptor-ligated library

This step uses the components listed in Table 16. Thaw the reagents listed below and keep on ice.

Table 16 Reagents for pre-capture PCR amplification

Component	Storage Location
SureSelect Primer	SureSelect XT Library Prep Kit ILM, –20°C
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C
Herculase II Fusion DNA Polymerase	Herculase II Fusion DNA Polymerase kit, –20°C
5× Herculase II Reaction Buffer	Herculase II Fusion DNA Polymerase kit*, –20°C
100 mM dNTP Mix	Herculase II Fusion DNA Polymerase kit*, –20°C

* *Do not use the PCR Reaction Buffer or dNTP mix from any other kit.*

This protocol uses half of the adaptor-ligated library for amplification. The remainder can be saved at –20°C for future use, if needed.

CAUTION To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

1 Prepare the appropriate volume of pre-capture PCR reaction mix, as described in Table 17, on ice. Mix well on a vortex mixer.

Table 17 Preparation of SureSelect Pre-Capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	21 µL	346.5 µL
SureSelect Primer (brown cap)	1.25 µL	20.6 µL
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer (clear cap)	1.25 µL	20.6 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	165 µL
100 mM dNTP Mix (green cap)	0.5 µL	8.25 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	16.5 µL
Total	**35 µL**	**577.5 µL**

2 Combine 35 µL of the PCR reaction mixture prepared in Table 17 and 15 µL of each purified DNA library sample from step 16 on page 33. Add a single DNA library sample to each well of the plate or strip tube.

Mix by pipetting.

3 Run the program in Table 18 in a thermal cycler.

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Table 18 Pre-Capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	4–6	98°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

NOTE

Different library preparations can produce slightly different results, based on varying DNA quality. In most cases, five cycles will produce an adequate yield for subsequent capture without introducing bias or non-specific products. If yield is too low, or too high (where non-specific high molecular weight products are observed), adjust the number of cycles accordingly to amplify the remaining library template.

Step 11. Purify the amplified library with AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL amplified DNA sample in the PCR plate. Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 30 µL nuclease-free water to each sample well.
13. Seal the wells, then mix well on a vortex mixer and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 30 µL) to a fresh PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 12. Assess quality and quantity

Quality assessment can be done with either the 2100 Bioanalyzer instrument or the 2200 TapeStation instrument.

Option 1: Analysis using the 2100 Bioanalyzer and DNA 1000 Assay

See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the DNA 1000 assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify that the electropherogram shows a distribution with a DNA fragment size peak of approximately 225 to 275 bp. Determine the concentration of the library DNA by integrating under the peak. A sample electropherogram is shown in Figure 4.

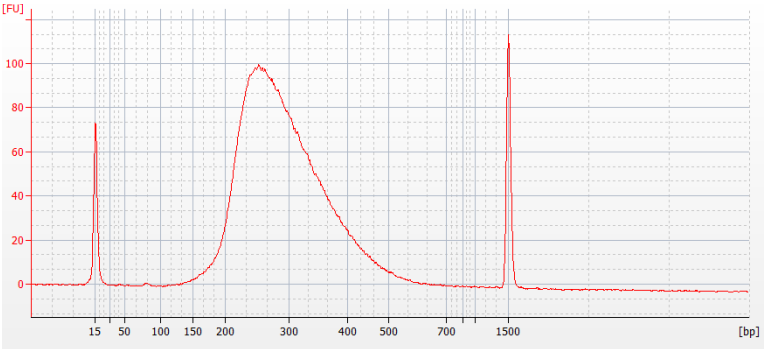

Figure 4 Analysis of amplified library DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using the 2200 TapeStation and D1000 ScreenTape

For more information to do this step, see the *Agilent 2200 TapeStation User Manual* at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the *Agilent 2200 TapeStation User Manual*. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the D1000 ScreenTape, and loading tips into the 2200 TapeStation as instructed in the *Agilent 2200 TapeStation User Manual*. Start the run.

3 Verify that the electropherogram shows a distribution with a DNA fragment size peak of approximately 225 to 275 bp. Determine the concentration of the library DNA by integrating under the peak. A sample electropherogram is shown in Figure 5.

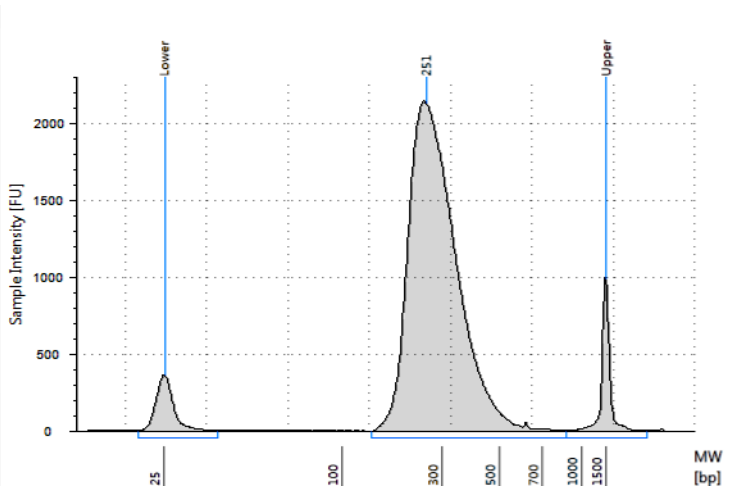

Figure 5 Analysis of amplified library DNA using a D1000 ScreenTape.

3 Sample Preparation (200 ng DNA Samples)

Step 1. Shear the DNA 42
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Step 9. Amplify the adaptor-ligated library 53
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CAUTION This section contains instructions for the preparation of gDNA libraries from 200 ng DNA samples. **For higher input (3 µg) DNA samples, see the library preparation protocol on page 21.**

The sample preparation protocol is used to prepare DNA libraries for sequencing using the Illumina paired-read platform. For each sample to be sequenced, an individual indexed library is prepared. For an overview of the SureSelect^{XT} target enrichment workflow, see Figure 1 on page 10.

The steps in this section differ from the Illumina protocol in the use of the Covaris system for gDNA shearing, smaller target shear size, elimination of size selection by gel purification, implementation of AMPure XP beads for all purification steps, and primers used for PCR. Refer to the Illumina protocol *Preparing Samples for Multiplexed Paired-End Sequencing* (p/n1005361) or the appropriate Illumina protocol for more information.

Step 1. Shear the DNA

NOTE Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0. Use the Qubit system to quantify genomic DNA before library preparation.

For each DNA sample to be sequenced, prepare 1 library.

1 Set up the Covaris E-series or S-series instrument.

   a Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate in use.

   b Check that the water covers the visible glass part of the tube.

   c On the instrument control panel, push the Degas button. Degas the instrument for least 30 minutes before use, or according to the manufacturer's recommendations.

   d Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C.

   e *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

   Refer to the Covaris instrument user guide for more details.

2 Put a Covaris microTube into the loading and unloading station.

   Keep the cap on the tube.

NOTE You can use the 96 microTube plate (see Table 6 on page 17) for the DNA shearing step when preparing multiple gDNA samples in the same experiment.

3 Use the Qubit dsDNA Assay to determine the concentration of your gDNA sample.

   Follow the instructions for the instrument.

4 Dilute 200 ng of high-quality gDNA with 1X Low TE Buffer in a 1.5-mL LoBind tube to a total volume of 50 µL.

5 Use a tapered pipette tip to slowly transfer the 50-µL DNA sample through the pre-split septa.

   Be careful not to introduce a bubble into the bottom of the tube.

6 Secure the microTube in the tube holder and shear the DNA with the settings in Table 19 or Table 20, depending on the Covaris instrument SonoLab software version used.

The target DNA fragment size is 150 to 200 bp.

Table 19 Shear settings for Covaris instruments using SonoLab software version 7 or newer

Setting	Value
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	200
Treatment Time	360 seconds
Bath Temperature	4° to 8° C

Table 20 Shear settings for Covaris instruments using SonoLab software prior to version 7

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Time	6 cycles of 60 seconds each
Set Mode	Frequency sweeping
Temperature	4° to 7° C

7 Put the Covaris microTube back into the loading and unloading station.

8 While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA.

9 Transfer each sheared DNA sample (approximately 50 µL) to a separate well of a 96-well plate or strip tube.

NOTE Instructions in this protocol are for sample processing in 96-well PCR plates. When processing a small number of samples, you can instead use strip tubes or individual tubes that are compatible with the thermal cycler and magnetic separation device used in the protocol.

Step 2. Assess quality (optional)

This step is optional.

Quality assessment can be done with the 2100 Bioanalyzer instrument.

For analysis of **200 ng sheared DNA samples, Use a High Sensitivity DNA chip and reagent kit.** See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the High Sensitivity DNA assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Check that the electropherogram shows a DNA fragment size peak between 120–150 bp. A sample electropherogram is shown in Figure 6.

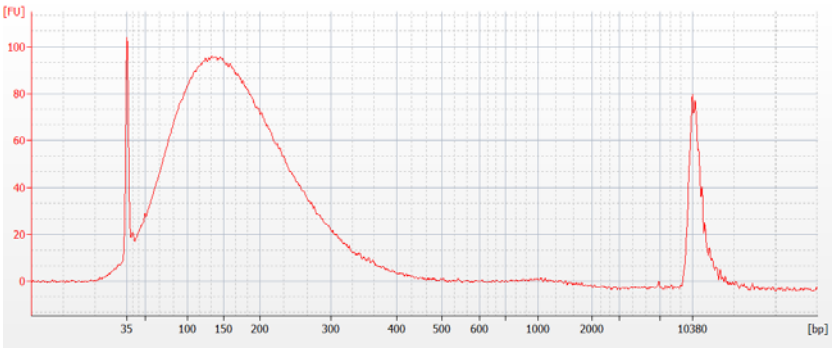

Figure 6 Analysis of sheared DNA using a High Sensitivity DNA Bioanalyzer assay.

Step 3. Repair the ends

Use the SureSelect XT Library Prep Kit ILM for this step.

To process multiple samples, prepare master mixes with overage at each step, without the DNA sample. Master mixes for preparation of 16 samples (including excess) are shown in each table as an example.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of End Repair master mix, as described in Table 21, on ice. Mix well on a vortex mixer.

Table 21 Preparation of End Repair master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	35.2 µL	580.8 µL
10× End Repair Buffer (clear cap)	10 µL	165 µL
dNTP Mix (green cap)	1.6 µL	26.4 µL
T4 DNA Polymerase (purple cap)	1 µL	16.5 µL
Klenow DNA Polymerase (yellow cap)	2 µL	33 µL
T4 Polynucleotide Kinase (orange cap)	2.2 µL	36.3 µL
Total	**52 µL**	**858 µL**

2 Add 52 µL of the master mix to each PCR plate well containing the sheared DNA samples (approximately 48–50 µL). Mix by pipetting up and down.

3 Incubate the plate in the thermal cycler and run the program in Table 22. Do not use a heated lid.

Table 22 End-Repair Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

Step 4. Purify the sample using AMPure XP beads

1 Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*

2 Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.

NOTE The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 1.6 mL of fresh 70% ethanol per sample.

3 Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.

4 Add 180 µL of homogeneous AMPure XP beads to each end-repaired DNA sample (approximately 100 µL) in the PCR plate. Pipette up and down 10 times to mix.

5 Incubate samples for 5 minutes at room temperature.

6 Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).

7 Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.

NOTE If some magnetic beads remain suspended in solution after 5 minutes, carefully remove and discard 100 µl of cleared solution from near the bottom of the wells, and continue incubating the plate in the magnetic stand for an additional 3 minutes. After the remaining suspension has cleared, remove and discard the remaining cleared solution (approximately 180 µl) from the wells.

8 Continue to keep the plate in the magnetic stand while you dispense 200 µL of 70% ethanol in each sample well.

Use fresh 70% ethanol for optimal results.

9 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.

10 Repeat step 8 to step 9 once.

11 Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

12 Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 3 to 5 minutes or until the residual ethanol completely evaporates.

NOTE Do not dry the bead pellet to the point that the pellet appears cracked during any of the bead drying steps in the protocol. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

13 Add 32 µL nuclease-free water to each sample well.

14 Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.

15 Incubate for 2 minutes at room temperature.

16 Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

17 Remove the cleared supernatant (approximately 30 µL) to a fresh PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 5. Adenylate the 3' end of the DNA fragments

Use the SureSelect XT Library Prep Kit ILM for this step.

Hold samples on ice while setting up this step.

1 Prepare the appropriate volume of Adenylation master mix, as described in Table 23, on ice. Mix well on a vortex mixer.

Table 23 Preparation of Adenylation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	11 µL	181.5 µL
10× Klenow Polymerase Buffer (blue cap)	5 µL	82.5 µL
dATP (green cap)	1 µL	16.5 µL
Exo(–) Klenow (red cap)	3 µL	49.5 µL
Total	**20 µL**	**330 µL**

2 Add 20 µL of the Adenylation master mix to each end-repaired, purified DNA sample (approximately 30 µL).

3 Mix well by pipetting up and down.

4 Incubate the plate in the thermal cycler and run the program in Table 24. Do not use a heated lid.

Table 24 dA-Tailing Thermal Cycler Program

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	4°C	Hold

Step 6. Purify the sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL dA-tailed DNA sample in the PCR plate. Pipette up and down 10 times to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 to step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 15 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove 13 µL of the cleared supernatant to a fresh PCR plate well. You can discard the beads at this time.
17. Proceed immediately to the next step, Step 7. Ligate the paired-end adaptor.

Step 7. Ligate the paired-end adaptor

Use the SureSelect XT Library Prep Kit ILM for this step.

Hold samples on ice while setting up this step.

1 Dilute the SureSelect Adaptor Oligo Mix (brown cap) 1:10 in nuclease-free water immediately before use. Use the diluted oligo mix when preparing the Ligation master mix in the next step.

2 Prepare the appropriate volume of Ligation master mix, as described in Table 25, on ice. Mix well on a vortex mixer.

Table 25 Preparation of Ligation master mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	15.5 µL	255.75 µL
5× T4 DNA Ligase Buffer (green cap)	10 µL	165 µL
Diluted SureSelect Adaptor Oligo Mix from step 1	10 µL	165 µL
T4 DNA Ligase (red cap)	1.5 µL	24.75 µL
Total	**37 µL**	**610.5 µL**

3 Add 37 µL of the Ligation master mix to each dA-tailed, purified DNA sample (13 µL) in the PCR plate wells.

4 Mix well by pipetting up and down.

5 Incubate the plate in the thermal cycler and run the program in Table 26. Do not use a heated lid.

Table 26 Ligation Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	4°C	Hold

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 8. Purify the sample using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each adaptor-ligated DNA sample in the PCR plate (50 µL). Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 32 µL nuclease-free water to each sample well.
13. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 30 µL) to a fresh PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 9. Amplify the adaptor-ligated library

This step uses the components listed in Table 27. Thaw the reagents listed below and keep on ice.

Table 27 Reagents for pre-capture PCR amplification

Component	Storage Location
SureSelect Primer (brown cap)	SureSelect XT Library Prep Kit ILM, –20°C
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer (clear cap)	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C
Herculase II Fusion DNA Polymerase (red cap)	Herculase II Fusion DNA Polymerase kit, –20°C
5× Herculase II Reaction Buffer (clear cap)	Herculase II Fusion DNA Polymerase kit*, –20°C
100 mM dNTP Mix (green cap)	Herculase II Fusion DNA Polymerase kit*, –20°C

* *Do not use the PCR Reaction Buffer or dNTP mix from any other kit.*

CAUTION To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

1 Prepare the appropriate volume of pre-capture PCR reaction mix, as described in Table 28, on ice. Mix well on a vortex mixer.

Table 28 Preparation of SureSelect Pre-Capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	6 µL	99.0 µL
SureSelect Primer (brown cap)	1.25 µL	20.6 µL
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer (clear cap)	1.25 µL	20.6 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	165 µL
100 mM dNTP Mix (green cap)	0.5 µL	8.3 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	16.5 µL
Total	**20 µL**	**330 µL**

2 Add 20 µL of the PCR reaction mixture prepared in Table 28 to each purified DNA library sample (30 µL) in the PCR plate wells.

Mix by pipetting.

3 Run the program in Table 29 in a thermal cycler.

.

Table 29 Pre-Capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10	98°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

Step 10. Purify the amplified library with AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
3. Add 90 µL of homogeneous AMPure XP beads to each 50-µL amplified DNA sample in the PCR plate. Pipette up and down to mix.
4. Incubate samples for 5 minutes at room temperature.
5. Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
6. Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
7. Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
8. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
9. Repeat step 7 and step 8 step once.
10. Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
11. Dry the samples by placing the unsealed plate on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
12. Add 30 µL nuclease-free water to each sample well.
13. Seal the wells, then mix well on a vortex mixer and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
14. Incubate for 2 minutes at room temperature.
15. Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
16. Remove the cleared supernatant (approximately 30 µL) to a fresh PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 11. Assess quality and quantity

Sample analysis can be done with either the 2100 Bioanalyzer instrument or the 2200 TapeStation instrument.

Option 1: Analysis using the 2100 Bioanalyzer and DNA 1000 Assay

See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
2. Open the 2100 Expert Software (version B.02.02 or higher), turn on the 2100 Bioanalyzer and check communication.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Within the instrument context, choose the DNA 1000 assay from the drop down list.
6. Start the run. Enter sample names and comments in the Data and Assay context.
7. Verify that the electropherogram shows a distribution with a DNA fragment size peak of approximately 225 to 275 bp. Determine the concentration of the library DNA by integrating under the peak. A sample electropherogram is shown in Figure 7.

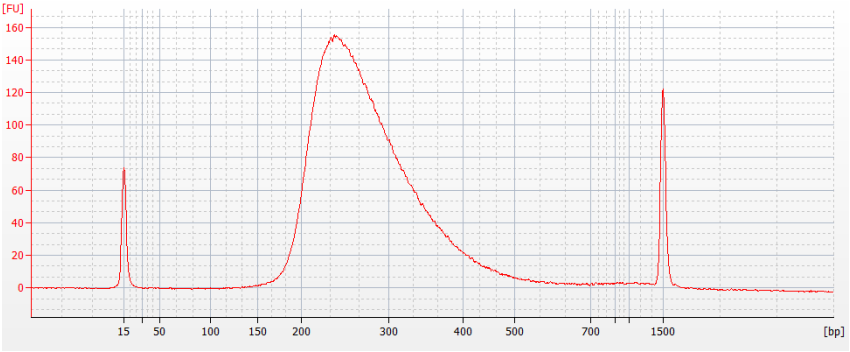

Figure 7 Analysis of amplified library DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using the 2200 TapeStation and D1000 ScreenTape

For more information to do this step, see the *Agilent 2200 TapeStation User Manual* at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the *Agilent 2200 TapeStation User Manual*. Use 1 µL of each DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the D1000 ScreenTape, and loading tips into the 2200 TapeStation as instructed in the *Agilent 2200 TapeStation User Manual*. Start the run.

3 Verify that the electropherogram shows a distribution with a DNA fragment size peak of approximately 225 to 275 bp. Determine the concentration of the library DNA by integrating under the peak. A sample electropherogram is shown in Figure 8.

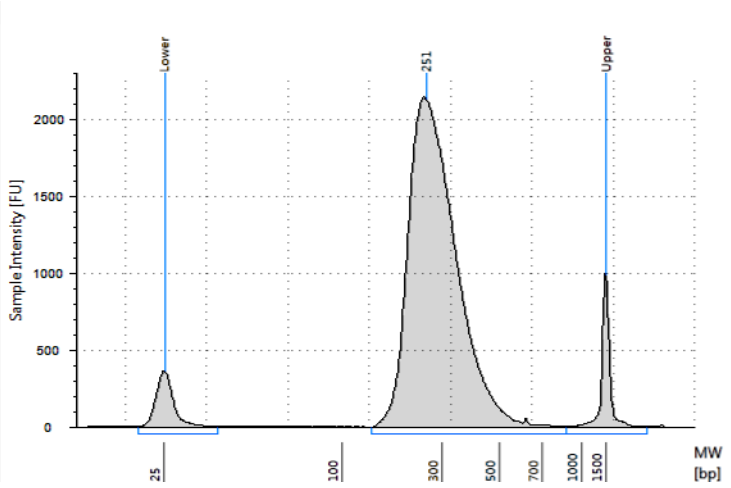

Figure 8 Analysis of amplified library DNA using a D1000 ScreenTape.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

4 Hybridization and Capture

Step 1. Hybridize DNA samples to the Capture Library 60
Step 2. Prepare streptavidin-coated magnetic beads 66
Step 3. Capture the hybridized DNA using streptavidin-coated beads 67

This chapter describes the steps to hybridize the prepared gDNA libraries with a target-specific Capture Library. After hybridization, the targeted molecules are captured on streptavidin beads.

Each DNA library sample must be hybridized and captured individually prior to addition of the indexing tag by PCR.

CAUTION The ratio of Capture Library to gDNA library is critical for successful capture.

Step 1. Hybridize DNA samples to the Capture Library

In this step, the prepared gDNA libraries are hybridized to a target-specific Capture Library.

This step uses the SureSelect^{XT} Reagent Kit components listed in Table 30. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 30 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect Hyb 1	SureSelect Target Enrichment-Box 1, RT	—	page 62
SureSelect Hyb 2	SureSelect Target Enrichment-Box 1, RT	—	page 62
SureSelect Hyb 3	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C	Warm to Room Temperature (RT)	page 62
SureSelect Hyb 4	SureSelect Target Enrichment-Box 1, RT	—	page 62
SureSelect Indexing Block 1	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C	Thaw on ice	page 62
SureSelect Block 2	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C	Thaw on ice	page 62
SureSelect ILM Indexing Block 3	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C	Thaw on ice	page 62
SureSelect RNase Block	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C	Thaw on ice	page 63
Capture Library	–80°C	Thaw on ice	page 64

For each sample library prepared, do one hybridization and capture. Do not pool samples at this stage.

The hybridization reaction requires 750 ng of prepared DNA in a volume of 3.4 µL (initial concentration of 221 ng/µL).

1 For prepped libraries with DNA concentrations above 221 ng/µL, prepare 3.4 µL of a 221 ng/µL dilution of each library.

2 For prepped libraries with DNA concentrations below 221 ng/µL, use a vacuum concentrator to concentrate the samples at ≤ 45°C.

   a Add the entire 30-µL volume of prepped library to an Eppendorf tube. Poke one or more holes in the lid with a narrow gauge needle.

   You can also break off the cap, cover with parafilm, and poke a hole in the parafilm.

   b Dehydrate using a vacuum concentrator on low heat (less than 45°C).

   c Reconstitute with nuclease-free water to a final concentration of 221 ng/µL. Pipette up and down along the sides of the tube for optimal recovery.

   d Mix well on a vortex mixer and spin in a centrifuge for 1 minute.

3 Transfer each 3.4-µL gDNA library sample (750 ng) to a separate well of a 96-well plate or strip tube. Seal the wells and keep on ice.

CAUTION

You must avoid evaporation from the small volumes of the capture during the 16 or 24 hour incubation.

If you want to use a different combination of thermal cycler, lid temperature, plates or strips, and sealing method (strip caps or sealing tape), first test the conditions. Incubate 27 µL of water at 65°C for 24 hours as a test. Include water in each well that you might use, including those in the center and those on the edges. Check that you do not get extensive evaporation. Evaporation should not exceed 4 µL.

For a partial list of tested options showing minimal evaporation, refer to "Alternative Capture Equipment Combinations" on page 97.

4 Prepare the Hybridization Buffer by mixing the components in Table 31 at room temperature.

If a precipitate forms, warm the Hybridization Buffer at 65°C for 5 minutes.

Keep the prepared Hybridization Buffer at room temperature until it is used in step 9.

Table 31 Preparation of Hybridization Buffer

Reagent	Volume for 1 reaction*	Volume for 16 reactions (includes excess)
SureSelect Hyb 1 (orange cap or bottle)	6.63 µL	116 µL
SureSelect Hyb 2 (red cap)	0.27 µL	4.7 µL
SureSelect Hyb 3 (yellow cap or bottle)	2.65 µL	46.4 µL
SureSelect Hyb 4 (black cap or bottle)	3.45 µL	60.4 µL
Total	**13 µL**	**227.5**

* Prepare Hybridization Buffer for at least 5 reaction equivalents per run to allow accurate pipetting volumes.

5 Prepare the SureSelect Block Mix by mixing the components in Table 32. Keep the mixture on ice until it is used in step 6.

Table 32 Preparation of SureSelect Block Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
SureSelect Indexing Block 1 (green cap)	2.5 µL	42.5 µL
SureSelect Block 2 (blue cap)	2.5 µL	42.5 µL
SureSelect ILM Indexing Block 3 (brown cap)	0.6 µL	10.2 µL
Total	**5.6 µL**	**95.2 µL**

CAUTION For each protocol step that requires removal of tube cap strips, make sure to reseal the tubes with a fresh strip of caps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during incubations.

6 To each gDNA library sample well prepared in step 3 on page 61, add 5.6 µL of the SureSelect Block Mix prepared in Table 32. Pipette up and down to mix.

7 Cap the wells, then transfer the sealed plate or strip tube to the thermal cycler and run the following program shown in Table 33.

Use a heated lid, set at 105°C, to hold the temperature at 65°C.

Make sure that the DNA + Block Mix samples are held at 65°C for at least 5 minutes before adding the remaining hybridization reaction components in step 10 below.

Table 33 Thermal cycler program for DNA + Block Mix prior to hybridization

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold (at least 5 minutes)

CAUTION The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.

8 Prepare the appropriate dilution of SureSelect RNase Block, based on the size of your Capture Library, according to Table 34. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Keep the mixture on ice until it is used in step 9.

Table 34 Preparation of RNase Block dilution

Capture Library Size	RNase Block dilution (parts RNase Block:parts water)	Volume of dilute RNase Block Required per hybridization reaction
≥3.0 Mb	25% (1:3)	2 µL
<3.0 Mb	10% (1:9)	5 µL

NOTE

Prepare the Capture Library mixture described in step 9, below, near the end of the 65°C hold step of >5 minute duration) described in Table 33. Keep the mixture at room temperature briefly, until adding the mixture to sample wells in step 10. Do not keep solutions containing the Capture Library at room temperature for extended periods.

9 Prepare the Capture Library Hybridization Mix appropriate for your Capture Library size according to Table 35 (Capture Libraries≥3 Mb), or Table 36 (Capture Libraries<3 Mb).

Mix well by vortexing at high speed for 5 seconds then spin down briefly. Keep the mixture at room temperature briefly, until use in step 10.

Table 35 Preparation of Capture Library Hybridization Mix for ≥**3 Mb Capture Libraries**

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Hybridization Buffer mixture from step 4	13 µL	221 µL
25% RNase Block solution from step 8	2 µL	34 µL
Capture Library ≥3 Mb	5 µL	85 µL
Total	**20 µL**	**340 µL**

Table 36 Preparation of Capture Library Hybridization Mix for **<3 Mb Capture Libraries**

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Hybridization Buffer mixture from step 4	13 µL	221 µL
10% RNase Block solution from step 8	5 µL	85 µL
Capture Library <3 Mb	2 µL	34 µL
Total	**20 µL**	**340 µL**

10 Maintain the gDNA library + Block Mix plate or strip tube at 65°C while you add 20 µL of the Capture Library Hybridization Mix from step 9 to each sample well. Mix well by pipetting up and down 8 to 10 times.

The hybridization reaction wells now contain approximately 27 to 29 µL, depending on the degree of evaporation during the thermal cycler incubation.

11 Seal the wells with strip caps or using the PlateLoc Thermal Microplate Sealer. Make sure that all wells are completely sealed.

CAUTION Wells must be adequately sealed to minimize evaporation, or your results can be negatively impacted.

When using the SureCycler 8800 thermal cycler and sealing with strip caps, make sure to use domed strip caps and to place a compression mat over the PCR plate or strip tubes in the thermal cycler.

12 Incubate the hybridization mixture for 16 or 24 hours at 65°C with a heated lid at 105°C.

Step 2. Prepare streptavidin-coated magnetic beads

The hybrid capture protocol uses reagents provided in SureSelect Target Enrichment Box 1 (stored at room temperature) in addition to the streptavidin-coated magnetic beads obtained from another supplier (see Table 2 on page 14).

1. Prewarm SureSelect Wash Buffer 2 at 65°C in a circulating water bath or heat block for use in "Step 3. Capture the hybridized DNA using streptavidin-coated beads" on page 67.
2. Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The magnetic beads settle during storage.
3. For each hybridization sample, add 50 µL of the resuspended beads to wells of a fresh PCR plate or strip tube.
4. Wash the beads:
   a. Add 200 µL of SureSelect Binding Buffer.
   b. Mix by pipetting up and down until beads are fully resuspended.
   c. Put the plate or strip tube into a magnetic separator device.
   d. Wait until the solution is clear, then remove and discard the supernatant.
   e. Repeat step a through step d two more times for a total of 3 washes.
5. Resuspend the beads in 200 µL of SureSelect Binding Buffer.

NOTE

If you are equipped for higher-volume magnetic bead captures, the streptavidin beads may be batch-washed in an Eppendorf tube or conical vial. Start the batch-washing procedure using excess bead solution. After resuspending the washed beads in the appropriate volume of SureSelect Binding Buffer, aliquot 200 µl of the washed beads to plate or strip tube wells to be used for hybridization capture.

Step 3. Capture the hybridized DNA using streptavidin-coated beads

1 Estimate and record the volume of hybridization solution that remains after the 24 hour incubation.

2 Maintain the hybridization plate or strip tube at 65°C while you use a multichannel pipette to transfer the entire volume (approximately 25 to 29 µL) of each hybridization mixture to the plate or strip tube wells containing 200 µL of washed streptavidin beads.

   Mix well by slowly pipetting up and down until beads are fully resuspended.

NOTE Excessive evaporation, such as when less than 20 µL remains after hybridization, can indicate suboptimal capture performance. See Table 58 on page 97 for tips to minimize evaporation.

3 Cap the wells, then incubate the capture plate or strip tube on a Nutator mixer or equivalent for 30 minutes at room temperature.

   Make sure the samples are properly mixing in the wells.

4 Briefly spin the plate or strip tube in a centrifuge or mini-plate spinner.

5 Put the plate or strip tube in a magnetic separator to collect the beads. Wait until the solution is clear, then remove and discard the supernatant.

6 Resuspend the beads in 200 µL of SureSelect Wash Buffer 1. Mix by pipetting up and down until beads are fully resuspended.

7 Incubate the samples for 15 minutes at room temperature.

8 Briefly spin in a centrifuge or mini-plate spinner.

9 Put the plate or strip tube in the magnetic separator. Wait for the solution to clear, then remove and discard the supernatant.

CAUTION

It is important to maintain bead suspensions at 65°C during the washing procedure below to ensure specificity of capture.

Make sure that the SureSelect Wash Buffer 2 is pre-warmed to 65°C before use.

Do not use a tissue incubator, or other devices with significant temperature fluctuations, for the incubation steps.

10 Wash the beads with SureSelect Wash Buffer 2:

- **a** Resuspend the beads in 200 µL of 65°C prewarmed Wash Buffer 2. Pipette up and down until beads are fully resuspended.
- **b** Cap the wells, then incubate the sample plate or strip tube for 10 minutes at 65°C on the thermal cycler.
- **c** Put the plate or strip tube in the magnetic separator. Wait for the solution to clear, then remove and discard the supernatant.
- **d** Repeat step a through step c for a total of 3 washes.

  Make sure all of the wash buffer has been removed during the final wash.

11 Add 30 µL of nuclease-free water to each sample well. Pipette up and down to resuspend the beads.

Keep the samples on ice until they are used on page 71.

NOTE

Captured DNA is retained on the streptavidin beads during the post-capture amplification step.

5 Indexing and Sample Processing for Multiplexed Sequencing

Step 1A. Amplify the captured libraries with indexing primers containing 8-bp indexes A01–H12 70
Step 1B. Amplify the captured libraries with indexing primers containing 6-bp indexes 1–16 74
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Step 3. Assess indexed library DNA quantity and quality 79
Step 4. Quantify each index-tagged library by QPCR (optional) 83
Step 5. Pool samples for multiplexed sequencing 84
Guidelines for sequencing sample preparation and run setup 86

This chapter describes the steps to add index tags by amplification, and to purify and assess quality and quantity of the captured libraries. Sample pooling instructions are provided to prepare the indexed samples for multiplexed sequencing.

Note that Step 1A and Step 1B are alternative protocols. Follow the protocol appropriate for the set of indexing primers provided with your kit. Use Step 1A if your kit includes the reconfigured 8-bp indexing primers A01 to H12, provided in white-capped tubes (16 reaction kits) or a blue plate (96 reaction kits). Use Step 1B if your kit includes the original 6-bp indexing primers 1 to 16, provided in clear-capped tubes.

CAUTION

This chapter includes instructions for kits containing two different sets of indexing primers. **Verify that you are referencing the information appropriate for your kit version before you proceed.**

Step 1A covers kits with 8-bp indexes supplied in Library Prep Kit p/n 5500-0132 or 5500-0133 in format of white-capped tubes or blue plate (typically received December, 2014 or later).

Step 1B covers kits with 6-bp indexes supplied in Library Prep Kit p/n 5500-0105 or 5500-0075 in format of clear-capped tubes (typically received before December, 2014).

Step 1A. Amplify the captured libraries with indexing primers containing 8-bp indexes A01–H12

In this step, the SureSelect-enriched DNA libraries are PCR amplified in PCR reactions that include the appropriate indexing primer for each sample.

This step uses the components listed in Table 37. Thaw then vortex to mix the reagents listed below and keep on ice.

Table 37 Reagents for post-capture indexing by PCR amplification

Kit Component	Storage Location
5× Herculase II Reaction Buffer	Herculase II Fusion DNA Polymerase kit*, –20°C
100 mM dNTP Mix (25 mM each dNTP)	Herculase II Fusion DNA Polymerase kit*, –20°C
Herculase II Fusion DNA Polymerase	Herculase II Fusion DNA Polymerase kit, –20°C
SureSelect ILM Indexing Post-Capture Forward PCR Primer	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C
SureSelect 8 bp Indexes (reverse primers)	SureSelect XT Library Prep Kit ILM, –20°C

* *Do not use the PCR Reaction Buffer or dNTP mix from any other kit.*

Prepare one indexing amplification reaction for each DNA library.

CAUTION To avoid cross-contaminating libraries, set up PCR mixes in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

1 Determine the appropriate index assignments for each sample. See Table 52 in the "Reference" chapter for sequences of the index portion of the SureSelect 8 bp Indexes A01 through H12 indexing primers used to amplify the DNA libraries in this step.

   Use a different indexing primer for each sample to be sequenced in the same lane.

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 38, on ice. Mix well on a vortex mixer.

Table 38 Preparation of post-capture PCR Reaction mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	18.5 µL	314.5 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	170 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17 µL
100 mM dNTP Mix (green cap)	0.5 µL	8.5 µL
SureSelect ILM Indexing Post-Capture Forward PCR Primer (orange cap)	1 µL	17 µL
Total	**31 µL**	**527 µL**

3 Add 31 µL of the PCR reaction mix prepared in Table 38 to each sample well of a fresh PCR plate or strip tube.

4 Add 5 µL of the appropriate indexing primer (SureSelect 8 bp Indexes A01 through H02, provided in white-capped tubes *or* A01 through H12, provided in blue plate) to each well. Add only one of the 16 or 96 possible indexing primers to each reaction well.

5 Add the DNA library samples to the PCR reactions:

a Obtain the PCR plate or strip tube containing 30 µL of bead-bound target-enriched DNA samples from ice (prepared on page 68).

b Pipette each DNA sample up and down until the bead suspension is homogeneous, then transfer 14 µL of the sample to the appropriate well of the PCR plate or strip tube containing PCR reaction mix and indexing primer.

c Mix the PCR reactions well by pipetting.

d Store the remaining library-bound beads at –20°C for future use, if needed.

6 Transfer the PCR plate or strip tube to a thermal cycler and run the PCR amplification program shown in Table 39.

Table 39 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10 to 16 Cycles See Table 40 for recommendations based on Capture Library size	98°C	30 seconds
		57°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

Table 40 Post-capture PCR cycle number recommendations

Capture Size	Cycles
1 kb up to 0.5 Mb	16 cycles
0.5 Mb up to 1.49 Mb	14 cycles
>1.5 Mb	12 cycles
All Exon and Exome libraries	10 to 12 cycles
OneSeq libraries (all designs)	10 cycles

7 When the PCR amplification program is complete, spin the plate or strip tube briefly.

Proceed to "Step 2. Purify the amplified captured libraries using AMPure XP beads" on page 77.

Step 1B. Amplify the captured libraries with indexing primers containing 6-bp indexes 1–16

In this step, the SureSelect-enriched DNA libraries are PCR amplified in PCR reactions that include the appropriate indexing primer for each sample.

This step uses the components listed in Table 41. Thaw then vortex to mix the reagents listed below and keep on ice.

Table 41 Reagents for post-capture indexing by PCR amplification

Kit Component	Storage Location
5× Herculase II Reaction Buffer	Herculase II Fusion DNA Polymerase kit*, –20°C
100 mM dNTP Mix (25 mM each dNTP)	Herculase II Fusion DNA Polymerase kit*, –20°C
Herculase II Fusion DNA Polymerase	Herculase II Fusion DNA Polymerase kit, –20°C
SureSelect ILM Indexing Post-Capture Forward PCR Primer	SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2, –20°C
SureSelect^{XT} Index reverse primers	SureSelect XT Library Prep Kit ILM, –20°C

* *Do not use the PCR Reaction Buffer or dNTP mix from any other kit.*

Prepare one indexing amplification reaction for each DNA library.

CAUTION To avoid cross-contaminating libraries, set up PCR mixes in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

1 Determine the appropriate index assignments for each sample. See Table 57 in the "Reference" chapter for sequences of the index portion of the SureSelect PCR Primers Index 1 through Index 16 used to amplify the DNA libraries in this step.

   Use a different indexing primer for each sample to be sequenced in the same lane.

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 42, on ice. Mix well on a vortex mixer.

Table 42 Preparation of post-capture PCR Reaction mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	22.5 µL	382.5 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	170 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17 µL
100 mM dNTP Mix (green cap)	0.5 µL	8.5 µL
SureSelect ILM Indexing Post-Capture Forward PCR Primer (orange cap)	1 µL	17 µL
Total	**35 µL**	**595 µL**

3 Add 35 µL of the PCR reaction mix prepared in Table 42 to each sample well of a fresh PCR plate or strip tube.

4 Add 1 µL of the appropriate SureSelect PCR Primer Index 1 through Index 16 (provided in clear-capped tubes). Add only one of the 16 possible indexing primers to each reaction well.

5 Add the DNA library samples to the PCR reactions:

- **a** Obtain the PCR plate or strip tube containing 30 µL of bead-bound target-enriched DNA samples from ice (prepared on page 68).
- **b** Pipette each DNA sample up and down until the bead suspension is homogeneous, then transfer 14 µL of the sample to the appropriate well of the PCR plate or strip tube containing PCR reaction mix and indexing primer.
- **c** Mix the PCR reactions well by pipetting.
- **d** Store the remaining library-bound beads at –20°C for future use, if needed.

6 Transfer the PCR plate or strip tube to a thermal cycler and run the PCR amplification program shown in Table 39.

Table 43 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10 to 16 Cycles	98°C	30 seconds
	See Table 44 for recommendations based on Capture Library size	57°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

Table 44 Post-capture PCR cycle number recommendations

Capture Size	Cycles
1 kb up to 0.5 Mb	16 cycles
0.5 Mb up to 1.49 Mb	14 cycles
>1.5 Mb	12 cycles
All Exon and Exome libraries	10 to 12 cycles
OneSeq libraries (all designs)	10 cycles

7 When the PCR amplification program is complete, spin the plate or strip tube or strip tube briefly.

Proceed to "Step 2. Purify the amplified captured libraries using AMPure XP beads" on page 77.

Step 2. Purify the amplified captured libraries using AMPure XP beads

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
2. Prepare 400 µL of fresh 70% ethanol per sample, plus excess, for use in step 9.
3. Mix the AMPure XP bead suspension well so that the suspension appears homogeneous and consistent in color.
4. Add 90 µL of the homogeneous AMPure XP bead suspension to each 50-µL amplified DNA sample bead suspension in the PCR plate or strip tube.
5. Mix thoroughly by pipetting up and down.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.
6. Incubate samples for 5 minutes at room temperature.
7. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).
8. While keeping the plate or tubes in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not disturb the beads while removing the solution.
9. Continue to keep the plate or tubes in the magnetic stand while you dispense 200 µL of freshly prepared 70% ethanol in each sample well.
10. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
11. Repeat step 9 and step 10 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.
12. Seal the wells with strip caps, then briefly spin to collect the residual ethanol. Return the plate or strip tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
13. Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, for 1 to 2 minutes or until the residual ethanol completely evaporates.
14. Add 30 µL of nuclease-free water to each sample well.

15 Seal the sample wells, then mix well on a vortex mixer and briefly spin to collect the liquid.

16 Incubate for 2 minutes at room temperature.

17 Put the plate or strip tube in the magnetic stand and leave for 2 minutes or until the solution is clear.

18 Remove the cleared supernatant (approximately 30 µL) to a fresh well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, store the libraries at 4°C for up to one week or at –20°C for longer periods.

Step 3. Assess indexed library DNA quantity and quality

Option 1: Analysis using the Agilent 2100 Bioanalyzer and High Sensitivity DNA Assay

Use the Bioanalyzer High Sensitivity DNA Assay to analyze the amplified indexed DNA. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

1. Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
3. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
4. Verify that the electropherogram shows the peak of DNA fragment size positioned between 250 and 350 bp. A sample electropherogram is shown in Figure 9.
5. Measure the concentration of each library by integrating under the entire peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

If you wish to more-precisely quantify the target enriched samples prior to pooling, proceed to "Step 4. Quantify each index-tagged library by QPCR (optional)" on page 83.

Otherwise, proceed to "Step 5. Pool samples for multiplexed sequencing" on page 84.

Stopping Point If you do not continue to the next step, store the libraries at 4°C for up to one week or at –20°C for longer periods.

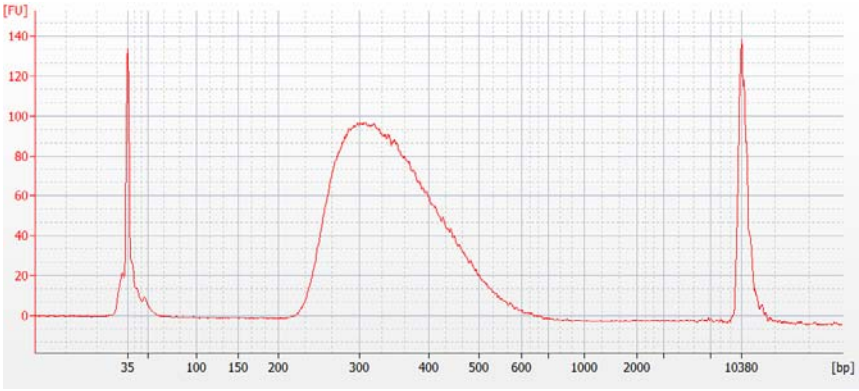

Figure 9 Post-capture analysis of amplified indexed library DNA using the 2100 Bioanalyzer and a High Sensitivity DNA Assay.

Option 2: Analysis using the Agilent 2200 TapeStation and High Sensitivity D1000 ScreenTape

Use a High Sensitivity D1000 ScreenTape (p/n 5067-5584) and reagent kit (p/n 5067-5585) to analyze the amplified indexed DNA. For more information to do this step, see the *Agilent 2200 TapeStation User Manual* at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the *Agilent 2200 TapeStation User Manual*. Use 2 µL of each indexed DNA sample diluted with 2 µL of High Sensitivity D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

2 Load the sample plate or tube strips from step 1, the High Sensitivity D1000 ScreenTape, and loading tips into the 2200 TapeStation as instructed in the *Agilent 2200 TapeStation User Manual*. Start the run.

3 Verify that the electropherogram shows the peak of DNA fragment size positioned between 250 and 350 bp. A sample electropherogram is shown in Figure 10.

4 Measure the concentration of each library by integrating under the entire peak.

If you wish to more-precisely quantify the target enriched samples prior to pooling, proceed to "Step 4. Quantify each index-tagged library by QPCR (optional)" on page 83.

Otherwise, proceed to "Step 5. Pool samples for multiplexed sequencing" on page 84.

Stopping Point If you do not continue to the next step, store the libraries at 4°C overnight or at –20°C for up to one month.

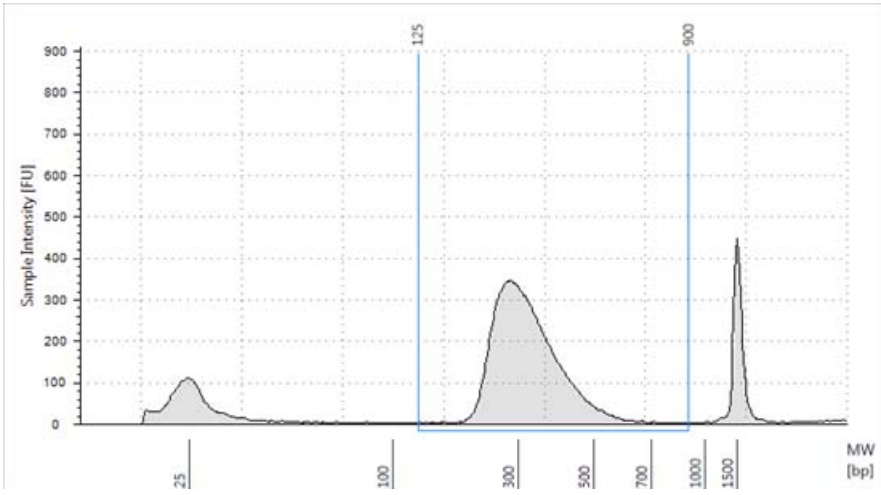

Figure 10 Post-capture analysis of amplified indexed library DNA using the 2200 TapeStation with a High Sensitivity D1000 ScreenTape.

Step 4. Quantify each index-tagged library by QPCR (optional)

You can use the Agilent QPCR NGS Library Quantification Kit (for Illumina) to accurately determine the concentration of each index-tagged captured library. Refer to the protocol that is included with the Agilent QPCR NGS Library Quantification Kit (p/n G4880A) for more details to do this step.

1 Prepare a standard curve using the quantification standard included in the kit, according to the instructions provided in the user guide.

2 Dilute each index-tagged captured library such that it falls within the range of the standard curve.

  Typically this corresponds to approximately a 1:1000 to 1:10,000 dilution of the captured DNA.

3 Prepare the QPCR master mix with Illumina adaptor-specific PCR primers according to instructions provided in the kit.

4 Add an aliquot of the master mix to PCR tubes and add template.

5 On a QPCR system, such as the Mx3005p, run the thermal profile outlined in the QPCR NGS Library Quantification kit user guide. Use the SYBR Green instrument setting.

6 Use the standard curve to determine the concentration of each unknown index-tagged library, in nM.

Step 5. Pool samples for multiplexed sequencing

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

1 Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool. For each library, use the formula below to determine the amount of indexed sample to use.

$$\text{Volume of Index} = \frac{V(f) \times C(f)}{\# \times C(i)}$$

where *V(f)* is the final desired volume of the pool,

C(f) is the desired final concentration of all the DNA in the pool

is the number of indexes, and

C(i) is the initial concentration of each indexed sample.

Table 45 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM.

Table 45 Example of indexed sample volume calculation for total volume of 20 µL

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	20 µL	20 nM	10 nM	4	2.5
Sample 2	20 µL	10 nM	10 nM	4	5
Sample 3	20 µL	17 nM	10 nM	4	2.9
Sample 4	20 µL	25 nM	10 nM	4	2
Low TE					7.6

2 Adjust the final volume of the pooled library to the desired final concentration.

- If the final volume of the combined index-tagged samples is less than the desired final volume, V(f), add Low TE to bring the volume to the desired level.
- If the final volume of the combined index-tagged samples is greater than the final desired volume, V(f), lyophilize and reconstitute to the desired volume.

3 If you store the library before sequencing, add Tween 20 to 0.1% v/v and store at -20°C short term.

Guidelines for sequencing sample preparation and run setup

Use the appropriate Illumina Paired-End Cluster Generation Kit to do cluster amplification.

Refer to the instructions that are included with the Illumina Paired-End Cluster Generation Kit. The optimal seeding concentration for SureSelect^{XT} libraries is 6 to 8 pM, depending on the desired output and data quality.

Sequencing run setup guidelines for 8-bp indexes

For libraries prepared using kits with 8-bp indexes, sequencing runs must be set up to perform an 8-bp index read. For the HiSeq platform, use the *Cycles* settings shown in Table 46. Cycle number settings can be specified on the *Run Configuration* screen of the instrument control software interface after choosing *Custom* from the index type selection buttons.

For complete 8-bp index sequence information, see Table 52 on page 92.

Table 46 HiSeq platform Run Configuration screen Cycle Number settings*

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	9
Index 2 (i5)	0
Read 2	100

* Settings apply to v3.0 SBS chemistry.

6 Reference

Reference Information for Kits with Revised Index Configuration (indexing primers in white-capped tubes or blue plate) 88
Reference Information for Kits with Original Index Configuration (indexing primers in clear-capped tubes) 93
Alternative Capture Equipment Combinations 97

This chapter contains reference information, including component kit contents and index sequences.

CAUTION This chapter contains two sets of index sequence and kit content information. The first section covers kits with 8-bp indexes supplied in Library Prep Kit p/n 5500-0132 or 5500-0133 (typically received December, 2014 or later). The second section covers kits with 6-bp indexes supplied in Library Prep Kit p/n 5500-0105 or 5500-0075 (typically received before December, 2014). **Verify that you are referencing the information appropriate for your kit version before you proceed.**

Reference Information for Kits with Revised Index Configuration (indexing primers in white-capped tubes or blue plate)

Use the reference information in this section if your kit includes Library Prep Kit p/n 5500-0132 or 5500-0133. If your kit does not include one of these component kits, see page 93 for kit content and indexing primer information.

Kit Contents

Each SureSelect^{XT} Reagent Kit contains the following component kits:

Table 47 SureSelect^{XT} Reagent Kit Contents-Revised Index Configuration

Product	Storage Condition	16 Reactions	96 Reactions	480 Reactions
SureSelect XT Library Prep Kit ILM	–20°C	5500-0132	5500-0133	5 x 5500-0133
SureSelect Target Enrichment-Box 1	Room Temperature	5190-8645	5190-8646	5 x 5190-8646
SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2	–20°C	5190-4455	5190-4456	5190-4457

NOTE SureSelect capture libraries and reagents must be used within one year of receipt.

The contents of each of the component kits listed in Table 47 are described in the tables below.

Table 48 SureSelect XT Library Prep Kit ILM Content-Revised Index Configuration

Kit Component	16 Reactions	96 or 480 Reactions
10X End Repair Buffer	tube with clear cap	tube with clear cap
10X Klenow Polymerase Buffer	tube with blue cap	tube with blue cap
5X T4 DNA Ligase Buffer	tube with green cap	tube with green cap
T4 DNA Ligase	tube with red cap	tube with red cap
Exo(-) Klenow	tube with red cap	tube with red cap
T4 DNA Polymerase	tube with purple cap	tube with purple cap
Klenow DNA Polymerase	tube with yellow cap	tube with yellow cap
T4 Polynucleotide Kinase	tube with orange cap	tube with orange cap
dATP	tube with green cap	tube with green cap
dNTP Mix	tube with green cap	tube with green cap
SureSelect Adaptor Oligo Mix	tube with brown cap	tube with brown cap
SureSelect Primer (forward primer)	tube with brown cap	tube with brown cap
SureSelect^{XT} 8 bp Index reverse primers*	SureSelect 8bp Indexes A01 through H02, provided in 16 white-capped tubes	SureSelect 8bp Indexes A01 through H12, provided in blue 96-well plate†

* See Table 52 on page 92 for index sequences.

† See Table 51 on page 91 for a plate map.

Table 49 SureSelect Target Enrichment-Box 1 Content

Kit Component	16 Reactions	96 or 480 Reactions
SureSelect Hyb 1	tube with orange cap	tube with orange cap
SureSelect Hyb 2	tube with red cap	tube with red cap
SureSelect Hyb 4	tube with black cap	tube with black cap
SureSelect Binding Buffer	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle

Table 50 SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2 Content

Kit Component	16 Reactions	96 Reactions	480 Reactions
SureSelect Hyb 3	tube with yellow cap	tube with yellow cap	bottle
SureSelect Indexing Block 1	tube with green cap	tube with green cap	tube with green cap
SureSelect Block 2	tube with blue cap	tube with blue cap	tube with blue cap
SureSelect ILM Indexing Block 3	tube with brown cap	tube with brown cap	tube with brown cap
SureSelect RNase Block	tube with purple cap	tube with purple cap	tube with purple cap
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer	tube with clear cap	tube with clear cap	tube with clear cap
SureSelect ILM Indexing Post-Capture Forward PCR Primer	tube with orange cap	tube with orange cap	tube with orange cap

Table 51 Plate map for SureSelect 8bp Indexes A01 through H12, provided in blue plate in Library Prep kit p/n 5500-0133

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
D	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
E	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
H	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Nucleotide Sequences of SureSelect^{XT} Indexes A01 to H12

Each index is 8 nt in length. See page 86 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 52 SureSelect^{XT} Indexes, for indexing primers provided in blue 96-well plate or white capped tubes

Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
A01	ATGCCTAA	A04	AACTCACC	A07	ACGTATCA	A10	AATGTTGC
B01	GAATCTGA	B04	GCTAACGA	B07	GTCTGTCA	B10	TGAAGAGA
C01	AACGTGAT	C04	CAGATCTG	C07	CTAAGGTC	C10	AGATCGCA
D01	CACTTCGA	D04	ATCCTGTA	D07	CGACACAC	D10	AAGAGATC
E01	GCCAAGAC	E04	CTGTAGCC	E07	CCGTGAGA	E10	CAACCACA
F01	GACTAGTA	F04	GCTCGGTA	F07	GTGTTCTA	F10	TGGAACAA
G01	ATTGGCTC	G04	ACACGACC	G07	CAATGGAA	G10	CCTCTATC
H01	GATGAATC	H04	AGTCACTA	H07	AGCACCTC	H10	ACAGATTC
A02	AGCAGGAA	A05	AACGCTTA	A08	CAGCGTTA	A11	CCAGTTCA
B02	GAGCTGAA	B05	GGAGAACA	B08	TAGGATGA	B11	TGGCTTCA
C02	AAACATCG	C05	CATCAAGT	C08	AGTGGTCA	C11	CGACTGGA
D02	GAGTTAGC	D05	AAGGTACA	D08	ACAGCAGA	D11	CAAGACTA
E02	CGAACTTA	E05	CGCTGATC	E08	CATACCAA	E11	CCTCCTGA
F02	GATAGACA	F05	GGTGCGAA	F08	TATCAGCA	F11	TGGTGGTA
G02	AAGGACAC	G05	CCTAATCC	G08	ATAGCGAC	G11	AACAACCA
H02	GACAGTGC	H05	CTGAGCCA	H08	ACGCTCGA	H11	AATCCGTC
A03	ATCATTCC	A06	AGCCATGC	A09	CTCAATGA	A12	CAAGGAGC
B03	GCCACATA	B06	GTACGCAA	B09	TCCGTCTA	B12	TTCACGCA
C03	ACCACTGT	C06	AGTACAAG	C09	AGGCTAAC	C12	CACCTTAC
D03	CTGGCATA	D06	ACATTGGC	D09	CCATCCTC	D12	AAGACGGA
E03	ACCTCCAA	E06	ATTGAGGA	E09	AGATGTAC	E12	ACACAGAA
F03	GCGAGTAA	F06	GTCGTAGA	F09	TCTTCACA	F12	GAACAGGC
G03	ACTATGCA	G06	AGAGTCAA	G09	CCGAAGTA	G12	AACCGAGA
H03	CGGATTGC	H06	CCGACAAC	H09	CGCATACA	H12	ACAAGCTA

Reference Information for Kits with Original Index Configuration (indexing primers in clear-capped tubes)

Use the reference information in this section if your kit includes Library Prep Kit p/n 5500-0105 or 5500-0075. If your kit does not include this component kit, see page 88 for kit content and indexing primer information.

Kit Contents

Each SureSelect^{XT} Reagent Kit contains the following component kits:

Table 53 SureSelect^{XT} Reagent Kit Contents-Original Index Configuration

Product	Storage Condition	16 Reactions	96 Reactions	480 Reactions
SureSelect XT Library Prep Kit ILM	–20°C	5500-0105	5500-0075	5 x 5500-0075
SureSelect Target Enrichment-Box 1	Room Temperature	5190-4393	5190-4394	5190-4395
SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2	–20°C	5190-4455	5190-4456	5190-4457

NOTE SureSelect capture libraries and reagents must be used within one year of receipt.

The contents of each of the component kits listed in Table 53 are described in the tables below.

Table 54 SureSelect XT Library Prep Kit ILM Content-Original Index Configuration

Kit Component	Format
10X End Repair Buffer	tube with clear cap
10X Klenow Polymerase Buffer	tube with blue cap
5X T4 DNA Ligase Buffer	tube with green cap
T4 DNA Ligase	tube with red cap
Exo(-) Klenow	tube with red cap
T4 DNA Polymerase	tube with purple cap
Klenow DNA Polymerase	tube with yellow cap
T4 Polynucleotide Kinase	tube with orange cap
dATP	tube with green cap
dNTP Mix	tube with green cap
SureSelect Adaptor Oligo Mix	tube with brown cap
SureSelect Primer (forward primer)	tube with brown cap
PCR Primer Index 1 through Index 16 (reverse primers containing 6-bp index sequences)*	16 tubes with clear caps

* See Table 57 on page 96 for index sequences.

Table 55 SureSelect Target Enrichment-Box 1 Content

Kit Component	16 Reactions	96 Reactions	480 Reactions
SureSelect Hyb 1	tube with orange cap	tube with orange cap	bottle
SureSelect Hyb 2	tube with red cap	tube with red cap	tube with red cap
SureSelect Hyb 4	tube with black cap	tube with black cap	bottle
SureSelect Binding Buffer	bottle	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle	bottle
SureSelect Elution Buffer*	tube with blue cap	bottle	bottle
SureSelect Neutralization Buffer*	bottle	bottle	bottle

* The provided SureSelect Elution Buffer and Neutralization Buffer are not used in the workflow described in this manual.

Table 56 SureSelect Target Enrichment Kit ILM Indexing Hyb Module Box 2 Content

Kit Component	16 Reactions	96 Reactions	480 Reactions
SureSelect Hyb 3	tube with yellow cap	tube with yellow cap	bottle
SureSelect Indexing Block 1	tube with green cap	tube with green cap	tube with green cap
SureSelect Block 2	tube with blue cap	tube with blue cap	tube with blue cap
SureSelect ILM Indexing Block 3	tube with brown cap	tube with brown cap	tube with brown cap
SureSelect RNase Block	tube with purple cap	tube with purple cap	tube with purple cap
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer	tube with clear cap	tube with clear cap	tube with clear cap
SureSelect ILM Indexing Post-Capture Forward PCR Primer	tube with orange cap	tube with orange cap	tube with orange cap

Nucleotide Sequences of SureSelect^{XT} 6-bp Indexes-Original Kit Configuration

Refer to the sequence information in Table 57 only if your kit includes Library Prep kit p/n 5500-0105 or p/n 5500-0075, with 6-bp indexing primers provided in 16 clear-capped tubes (original kit configuration).

Table 57 SureSelect^{XT} Indexes 1-16

Index Number	Sequence
1	ATCACG
2	CGATGT
3	TTAGGC
4	TGACCA
5	ACAGTG
6	GCCAAT
7	CAGATC
8	ACTTGA
9	GATCAG
10	TAGCTT
11	GGCTAC
12	CTTGTA
13	AAACAT
14	CAAAAG
15	GAAACC
16	AAAGCA

Alternative Capture Equipment Combinations

Table 58 below lists combinations of thermal cyclers, lid temperatures, plates or strip tubes and sealing methods that have shown minimal evaporation when used for the Hybridization protocol on page 60.

Refer to this list for additional equipment and plasticware combination options for hybridization, beyond the combinations used for protocol optimization and supported by Agilent. Note that minimal evaporation is required to ensure optimal capture results.

Table 58 Tested options that show minimal evaporation

PCR Machine	Plate/Strips	Cover	Comments
Agilent Mx3005P Real-Time PCR System	Mx3005P Strip Tubes (Agilent p/n 401428)	Mx3005P Optical Strip Caps (Agilent p/n 401425)	Heated lid
Agilent Mx3005P Real-Time PCR System	Life Technologies ABI MicroAmp Optical 96-well plates (p/n N8010560)	MicroAmp clear adhesive film (p/n 4306311)	Heated lid; use ABI compression pad (4312639); use two layers of film
ABI GeneAmp 9700	Life Technologies ABI MicroAmp Optical 96-well plates (p/n N8010560)	MicroAmp caps (p/n N8010535)	Heated lid
ABI Veriti (p/n 4375786)	Life Technologies ABI MicroAmp Optical 96-well plates (p/n N8010560)	MicroAmp clear adhesive film (p/n 4306311)	Heated lid; use ABI compression pad (4312639); use two layers of film
Eppendorf Mastercycler	Eppendorf 8-Tube PCR Tubes	Attached caps	Lid heating set to 75°C
BioRad (MJ Research) PTC-200	Mx4000 Strip Tubes (Agilent p/n 410022)	Mx4000 Optical Caps (Agilent p/n 401024)	Heated lid
BioRad (MJ Research) PTC-200	Mx4000 Strip Tubes (Agilent p/n 410022)	Mx3005P Optical Strip Caps (Agilent p/n 401425)	Heated lid
BioRad (MJ Research) PTC-200	Mx3005P 96-well plate (Agilent p/n 410088)	Mx3005P Optical Strip Caps (Agilent p/n 401425)	Heated lid

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In This Book

This guide contains information to run the SureSelect^{XT} target enrichment protocol.

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Version B.2, April 2015

p/n G7530-90000

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